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A potential endophenotype for Alzheimer's disease: cerebrospinal fluid clusterin

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ABSTRACT

Genome-wide association studies have associated clusterin (*CLU*) variants with Alzheimer's disease (AD). However, the role of CLU on AD pathogenesis is not totally understood. We used cerebrospinal fluid (CSF) and plasma CLU levels as endophenotypes for genetic studies to understand the role of CLU in AD. CSF, but not plasma, CLU levels were significantly associated with AD status and CSF tau/amyloid-beta ratio, and highly correlated with CSF apolipoprotein E (APOE) levels. Several loci showed almost genome-wide significant associations including *LINC00917* ($p = 3.98 \times 10^{-7}$) and interleukin 6 (*IL6*, $p = 9.94 \times 10^{-6}$, in the entire data set and in the *APOE* ε4- individuals $p = 7.40 \times 10^{-8}$). Gene ontology analyses suggest that CSF CLU levels may be associated with wound healing and immune response which supports previous functional studies that demonstrated an association between CLU and IL6. CLU may play a role in AD by influencing immune system changes that have been observed in AD or by disrupting healing after neurodegeneration.

1. Introduction

Clusterin (CLU), a multifunctional glycoprotein also known as apolipoprotein J, plays a role in several cellular processes including apoptosis, proliferation, and clearance of misfolded proteins (Kim and Choi, 2011; Wang et al., 2014; Wyatt et al., 2011). CLU is ubiquitous and is highly expressed in the brain by astrocytes (de Silva et al., 1990). CLU is a secreted protein, but some isoforms have been discovered in the cytoplasm and nucleus (Kimura et al., 1997; Leskov et al., 2003).

CLU was first associated with Alzheimer's disease (AD) in 1990 when it was found to be increased in the hippocampi of AD patients (May et al., 1990). Two independent genome-wide association studies (GWAS) in 2009 found rs11136000, an intronic variant in *CLU*, associated with AD (Lambert et al., $p = 3.7 \times 10^{-9}$ and Harold et al., $p = 8.5 \times 10^{-10}$) (Harold et al., 2009; Lambert et al., 2009). In a meta-analysis of the 2-stage GWAS by Harold et al., another single nucleotide polymorphism (SNP) in strong linkage disequilibrium (LD) with rs11136000 was also genome-wide significant, rs7982 ($r^2 = 0.95$). This SNP has 3 alleles resulting in either a synonymous or a missense variant located in exon 5 of *CLU* ($p = 8 \times 10^{-10}$) (Harold et al., 2009). In a meta-analysis of >74,000 individuals in

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[1] Data used in preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of ADNI investigators can be found at: http://adni.loni.usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf.

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Table 1
Characteristics of plasma and CSF ADNI and Knight ADRC data

	CSF (n = 673)		Plasma (n = 818)		CSF and plasma (n = 537)	
	ADRC	ADNI	ADRC	ADNI	ADRC	ADNI
Samples	400	273	312	506	305	232
Age in years (Mean ± SD)	73.03 ± 6.77	78.05 ± 6.71	73.22 ± 7.03	78.30 ± 7.40	73.25 ± 6.89	77.84 ± 6.97
Range	49–91	60–93	49–91	55–95	49–91	60–93
% AD cases	27.50	69.60	26.92	86.36	27.54	81.47
% Female	58.50	39.19	61.22	37.35	60.98	36.64
% APOE ε4	36.25	49.45	36.22	53.16	36.07	50.43

Key: AD, Alzheimer's disease; ADNI, Alzheimer's Disease Neuroimaging Initiative; ADRC, Alzheimer's Disease Research Center; APOE, apolipoprotein E; CSF, cerebrospinal fluid; SD, standard deviation.

2013, rs9331896, located in a *CLU* intron and also in strong LD with rs11136000 ($r^2 = 0.925$) and rs7982 ($r^2 = 0.889$), was also associated with AD ($p = 2.8 \times 10^{-25}$) (Lambert et al., 2013).

Increased cerebrospinal fluid (CSF) CLU levels combined with increased amyloid-beta (Aβ) levels were associated with increased entorhinal atrophy (Desikan et al., 2014), and elevated CSF CLU levels have been observed in AD patients (Nilselid et al., 2006). It has been suggested that CLU may play a role in AD, by interacting with apolipoprotein E (APOE) or alone, by affecting Aβ clearance and/or aggregation. $APOE^{-/-}$ and $CLU^{-/-}$ mice have similar Aβ levels, but in an APOE and/or CLU double knock-out mouse model, Aβ was significantly increased in CSF and brain interstitial fluid, suggesting that the APOE and CLU effects on Aβ levels are additive and not completely independent (DeMattos et al., 2004). CLU has high affinity for soluble Aβ (Ghiso et al., 1993) and associates specifically with $Aβ_{40}$ (Howlett et al., 2013). CLU levels also appear to affect aggregation of Aβ (Oda et al., 1995; Wilson et al., 2008).

We hypothesized that genetic variants associated with CSF or plasma CLU levels may also play a role in AD. We performed a single-stage GWAS to look for SNPs associated with CLU levels and followed up with gene ontology (GO) analyses to look at potential biological, cellular, and molecular categories that may be associated with plasma and CSF CLU levels. Differences in CSF CLU levels have been observed in AD patients versus controls and appear to affect Aβ levels. We hypothesized that CLU levels were also associated with CSF tau/Aβ ratio, a powerful predictor of cognitive decline that can be used to discriminate between vascular dementia and AD (de Jong et al., 2006; Fagan et al., 2007; Harari et al., 2014). Animal studies suggest that CLU and APOE have synergistic effects on Aβ levels and both have been associated with AD. Given this biology, we tested whether CLU and APOE levels in plasma or CSF are correlated.

2. Materials and methods

2.1. Ethics statement

The Institutional Review Board of all participating institutions approved the study. Written informed consent was obtained from participants or their family members.

2.2. Study participants

There were CSF CLU, APOE, tau, and Aβ levels from 673 unrelated individuals (Table 1): 400 from the Charles F. and Joanne Knight Alzheimer's Disease Research Center (Knight ADRC) (151 AD cases, 249 cognitively normal controls) and 273 from the Alzheimer's Disease Neuroimaging Initiative (ADNI) (205 cases, 68 controls). There were 818 individuals with plasma analyte levels (Table 1): 312 from Knight ADRC (124 cases, 188 controls) and 506 from ADNI (434 cases, 72 controls). In the combined data sets, 537 individuals had both CSF and plasma analyte levels (273 cases, 264 controls; Table 2). ADNI individuals were evaluated at the time of sample collection as described in the ADNI procedures manual (http://www.adni-info.org). Knight ADRC individuals were evaluated at the time of sample collection by Clinical Core personnel at Washington University; cases received a clinical diagnosis of AD in accordance with standard criteria and dementia severity was determined using the clinical dementia rating (Morris, 1993). Neuropsychological and clinical assessments and biological samples were collected for all participants as described previously (Cruchaga et al., 2011, 2013; Shaw et al., 2009; Toledo et al., 2011).

2.3. Genotyping and quality control

Knight ADRC samples were genotyped with the Illumina 610 or OmniExpress chip and ADNI samples with the Illumina 610 chip. A call rate of ≤98% for SNPs and individuals was applied, and SNPs not in Hardy-Weinberg equilibrium ($p < 1 \times 10^{-6}$) or with macrophage-activating factor (MAF) <0.02 were excluded. Quality control (QC) steps were applied to each genotyping array separately. X-chromosome SNPs were analyzed to verify gender identification. Duplicate and related individuals were found using pairwise genome-wide estimates of proportion identity-by-descent and eliminated from the analysis. We used EIGENSTRAT (Price et al., 2006) to calculate principal component factors for each sample and confirm ethnicity. Imputation was performed as described before (Cruchaga et al., 2013). Briefly, the 1000 Genome Data and BEAGLE v3.3.1 (Browning and Browning, 2007) software were used to impute up to 6 million SNPs. SNPs with a call rate <95% or a

Table 2
Characteristics of plasma and CSF data by disease status

	CSF (n = 673)		Plasma (n = 818)		CSF & plasma (n = 537)	
	Cases	Controls	Cases	Controls	Cases	Controls
Samples	300	373	521	297	273	264
Age in years (Mean ± SD)	76.68 ± 7.18	73.77 ± 6.92	77.65 ± 7.51	74.12 ± 7.44	76.88 ± 7.23	73.53 ± 6.94
Range	60–92	49–93	55–94	49–95	59–94	49–95
% Female	40.33	58.98	38.58	60.27	39.93	61.36
% APOE ε4	59.00	27.61	58.93	25.25	58.97	25.00

Key: APOE, apolipoprotein E; CSF, cerebrospinal fluid; SD, standard deviation.

BEAGLE $r^2 \leq 0.3$ were removed, leaving a total of 5,970,354 imputed and genotyped variants.

2.4. Analyte measurements and QC

Samples were measured for CLU and APOE protein levels, among others, by Rules-Based Medicine, Inc (RBM) using multiplex immunoassay on the Human Discovery Multi-Analyte Profile (MAP) panel v1.0 (https://rbm.myriad.com/products-services/humanmap-services/human-discoverymap/). The Knight ADRC Biomarker Core and ADNI measured CSF $A\beta_{42}$, tau, and phosphotau ($ptau_{181}$) levels as described previously (Fagan et al., 2006; Shaw et al., 2009). Analytes with a call rate ≥90% passed QC. Before data sets were combined, outliers were removed and values were normalized by log transformation then standardized by series so the mean for each analyte was equal to zero.

2.5. Statistical analyses

SAS v9.2 for Linux (copyright 2008 by SAS Institute Inc) was used to combine the ADNI and Knight ADRC data sets, and the log-transformed, standardized values were tested for normality using the Shapiro-Wilk test. We used R v3.1.3 (Team, 2015) to perform linear regression to determine if CSF or plasma levels of CLU were influenced by age or gender. We used age, gender, and study as covariates when testing for association of CLU levels in plasma and CSF with AD status, CSF tau, CSF Aβ, and CSF tau/Aβ ratio. Pearson correlation was used to determine whether CLU and APOE levels in CSF and plasma were correlated.

CLU protein levels were tested for association using an additive model in PLINK v1.9 (Chang et al., 2015) (http://www.cog-genomics.org/plink2). Covariates used were study, age, gender, and 2 principal component factors for population structure. Bonferroni-corrected statistical significance was defined as $p < 5 \times 10^{-8}$, and $p < 1 \times 10^{-5}$ was considered suggestive association. The genomic inflation factor was 1, indicating no inflation because of population stratification. ANNOVAR version March 3, 2015 (Wang et al., 2010), SNAP version 2.2 SNP data set 1000 Genomes Pilot 1 population panel CEU (Johnson et al., 2008) (http://www.broadinstitute.org/mpg/snap), SNPnexus (http://www.snp-nexus.org, http://brainarray.mbni.med.umich.edu/Brainarray/Database/SearchSNP/), build GRCh37/hg19 (Dayem Ullah et al., 2012), and the NCBI Database of Single Nucleotide Polymorphisms (dbSNPs) Build ID: 142 (http://www.ncbi.nlm.nih.gov/SNP/) (Sherry et al., 2001) were used to perform SNP annotation.

2.6. GO over-representation analyses

Before analysis, we pruned GWAS SNPs using the clump function in PLINK v1.9 (Chang et al., 2015). Significance threshold for index and clumped SNPs was 1 to include all SNPs. SNPs were clumped if they were within 1 Mb and in LD with the index ($r^2 = 0.8$). Index SNPs were mapped to genes using a gene map created from the Table Browser tool on the UCSC genome browser using the Feb 2009 (GRCh37/hg19) assembly (http://www.genome.ucsc.edu/, accessed March 18, 2015) (Karolchik et al., 2004; Kent and Haussler, 2001; Kent et al., 2002). SNPs were mapped to a gene if they were located within 20 kB of that gene; if SNPs were mapped to more than 1 gene, all genes were included in the analysis. Genes were only counted once regardless of how many SNPs were mapped to the gene. Using a significance threshold $p < 1 \times 10^{-4}$, the CSF analysis included186 pruned SNPs in 117 genes, and there were 165 SNPs in 79 genes in the plasma analysis.

GO analyses were performed using the Protein Analysis THrough Evolutionary Relationships (PANTHER) statistical over-representation test v9.0 release 20150430 (http://www.go.pantherdb.org) (Mi et al., 2013; Thomas et al., 2006) which used data from the Gene Ontology Consortium (GOC) (http://www.geneontology.org, June 6, 2015), and the ConsensusPathDB (CPDB) over-representation gene set analysis release 30 which used GOC version GO_201501 released January 2015 (http://www.cpdb.molgen.mpg.de) (Kamburov et al., 2011). CPDB compares rates of GO term membership between the background and candidate sets of genes by the hypergeometric test, and the resulting p-values are corrected for multiple testing using the false discovery rate method. PANTHER uses a binomial distribution test to calculate over-representation of candidate genes, relative to the background, for different GO terms. PANTHER uses the Bonferroni model for multiple test correction which is highly conservative in this case because ontology terms include parent and child terms that are tested together in the analysis and are not independent at all. Because of this, we decided to use the PANTHER analyses without Bonferroni correction. Background gene sets came from each tool's default which included 18,043 genes for CPDB (based on the number of HGNC gene symbols annotated to at least 1 GO term) and 20,814 genes for PANTHER (based on the total number of genes in human database obtained from Ensembl in April 2014). Of the 117 genes in the CSF analysis, 105 genes were assigned to at least 1 GO term by CPDB and PANTHER recognized 116 genes (the number of genes assigned to at least 1 GO term was not reported by PANTHER). Of the 79 genes in the plasma analysis, PANTHER recognized all the genes and 77 were assigned to at least 1 GO term by CPDB. CPDB tested all 3 types of GO terms (biological, cellular, and molecular) in 1 analysis. PANTHER tested each GO term type separately, further separating genes that were manually assigned to that term based on experimental evidence from those assigned electronically based on bioinformatics algorithms. Categories with corrected $p < 0.05$ for CPDB and uncorrected $p < 8.33 \times 10^{-3}$ for PANTHER (based on the 6 separate tests) were considered significant.

Association List GO Annotator (ALIGATOR) was used to perform analysis of the nonpruned GWAS data as described previously with a few changes (Holmans et al., 2009). A list of significant SNPs was converted into a list of genes in which the SNPs lie (between the start of the first and end of the last exon as defined by NCBI build 37.3). Each gene was counted once regardless of how many significant SNPs it contained. Replicate gene lists generated by randomly sampling SNPs (to allow for varying numbers of SNPs within genes) were used to obtain empirical enrichment p-values for each gene set. A bootstrap method was used to correct for testing multiple nonindependent gene sets, and test whether the number of significantly enriched gene sets was higher than expected. Significant genes <1 MB apart and located in the same functional gene set were grouped into 1 signal, to correct for LD between nearby genes. Gene sets were only classed as being enriched if they carried at least 2 signals. Only genes defined as "protein coding" by NCBI were analyzed, a total of 17,233 genes for plasma and 17,690 genes for CSF. Significance threshold for SNPs was $p < 1 \times 10^{-4}$, resulting in 49 genes for plasma and 85 for CSF.

A large pathway set, covering as many areas of biology as possible, comprised GOC (downloaded July 26, 2013) (Ashburner et al., 2000; Harris et al., 2004), Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/, June 4, 2013) (Kanehisa et al., 2012), PANTHER v8.1 (June 4, 2013) (Mi et al., 2013), Mouse Genome Informatics (http://www.informatics.jax.org, August 9, 2013) (Bult et al., 2008), Reactome pathways (http://www.reactome.org, July 27, 2013) (Croft et al., 2014), Biocarta pathways from the Molecular Signatures Database v4.0 (http://www.broadinstitute.org/gsea/msigdb/index.jsp, July 28, 2013), and the NCI pathway interaction database (http://pid.nci.nih.gov/download.shtml, July 28, 2013) (Schaefer et al., 2009). We restricted analysis to 9016 categories containing 10 to 1000 genes (200 for GO, given its large size relative to the other sets).

3. Results

3.1. Covariate associations with CLU levels

We used linear regression to determine if CSF or plasma levels of CLU were influenced by age, gender, or *APOE* ε4 allele. CLU levels in CSF and plasma were significantly influenced by gender but with opposite effects ($p = 8.21 \times 10^{-5}$, beta = 0.152 and $p = 1.21 \times 10^{-11}$, beta = −0.235 respectively; Supplemental Table 1). CSF levels of CLU were strongly associated with age ($p = 2.88 \times 10^{-9}$, beta = 0.227), but there was no significant association between plasma CLU levels and age ($p = 0.142$, beta = −0.052; Supplemental Table 1). CSF and plasma CLU levels were not associated with *APOE* genotype ($p = 0.296$, beta = 0.04 and $p = 0.279$, beta = 0.038, respectively) or *APOE* ε4 carrier status ($p = 0.091$, beta = 0.065 and $p = 0.806$, beta = 0.009, respectively; Supplemental Table 1).

3.2. CSF and plasma CLU levels in AD cases versus controls

We used logistic and linear regression to determine if CSF or plasma levels of CLU were associated with AD status, CSF tau, CSF Aβ, or CSF tau/Aβ ratio. Age, gender, and study were included as covariates. CSF CLU levels were significantly higher in cases (defined by clinical dementia rating >0; mean 24.74 μg/mL, SD 9.7) than controls (mean 19.92 μg/mL, SD 6.92; $p = 0.027$, beta = −0.391, Fig. 1), and there was a significant association between CSF tau/Aβ ratio and CSF CLU levels ($p = 3.82 \times 10^{-8}$, beta = 0.218, Supplemental Fig. 1). When the data set was stratified by APOE ε4 carrier status, the association between CSF CLU levels and CSF tau/Aβ ratio was still significant (ε4+: $p = 1.03 \times 10^{-4}$, beta = 0.238; ε4−: $p = 3.66 \times 10^{-4}$, beta = 0.181; Supplemental Table 2).

We also compared levels of CLU between cases and controls in plasma. There was no significant difference between cases (mean 291.81 μg/mL, SD 70.54) and controls (mean 220.57 μg/mL, SD 68.70; $p = 0.222$, beta = 0.241), and there was no significant association with CSF tau/Aβ ratio ($p = 0.396$, beta = 0.035) in the whole data set or APOE ε4 stratified data sets (Supplemental Table S2). Together, these results suggest CSF CLU would be a more informative biomarker for AD than plasma CLU.

3.3. Correlation between CLU and APOE in CSF and plasma

Because previous functional studies linked CLU with APOE, we analyzed whether there was any correlation between CLU and APOE levels in CSF and plasma in the 537 individuals with both plasma and CSF levels (Supplemental Table 3). CLU levels in plasma and CSF were not correlated (r = 0.044, $p = 0.311$), similar to what we found previously for CSF and plasma APOE (Cruchaga et al., 2012).

We found a strong correlation between CSF levels of CLU and APOE (r = 0.702, $p < 2.2 \times 10^{-16}$, Fig. 2), but the correlation in plasma was weaker (r = 0.315, $p = 1.22 \times 10^{-13}$). Correlation between CSF CLU and CSF APOE was independent of AD status (cases: r = 0.708, $p < 2.2 \times 10^{-16}$; controls: r = 0.721, $p < 2.2 \times 10^{-16}$) and relative levels of CSF Aβ (threshold: ADNI = 192 ng/mL, Knight ADRC = 500 ng/mL; high Aβ: r = 0.682, $p < 2.2 \times 10^{-16}$; low Aβ: r = 0.724, $p < 2.2 \times 10^{-16}$). Correlation between CLU and APOE in the CSF was also independent of APOE genotype (ε4+: r = 0.703, $p < 2.2 \times 10^{-16}$; ε4−: r = 0.75, $p < 2.2 \times 10^{-16}$). We did not find an association between CSF and plasma CLU levels.

3.4. GWAS for CSF and plasma CLU

In a previous GWAS, we looked for genetic loci associated with CSF levels of CLU in an overall smaller data set (n = 574) by analyzing each study and performing a meta-analysis, and we found no genome-wide significant associations (Kauwe et al., 2014). Joint analyses provide more statistical power than the power that meta-analysis approaches (Skol et al., 2006). For this reason, we performed linear regression on all 673 individuals with CSF CLU levels from ADNI and Knight ADRC. There were no genome-wide significant hits, but several loci had *p*-values less than the suggestive threshold $p = 1 \times 10^{-5}$ (Table 2, Supplemental Fig. 4). The most

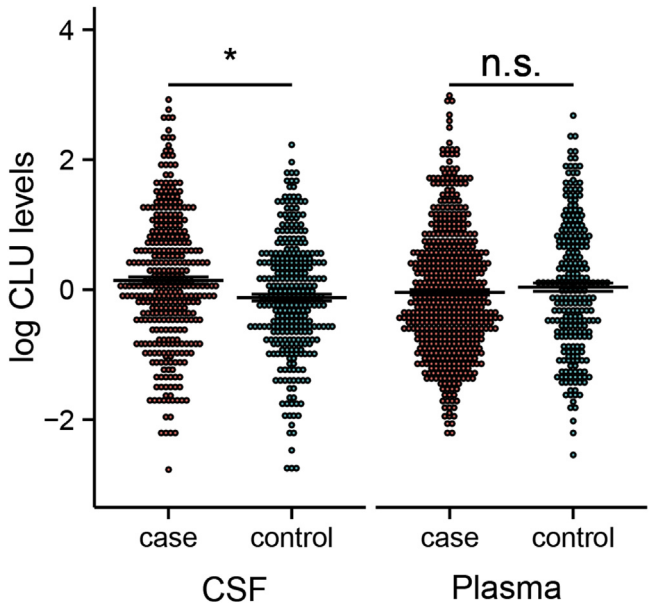

Fig. 1. Scatterplots of log normalized CLU levels in CSF and plasma in AD cases and controls. Error bars (mean ± SEM). CSF (cases = 24.74 ± 0.56 μg/mL, controls = 19.92 ± 0.36 μg/mL, $p = 0.027$), plasma (cases = 285.05 ± 3.12 μg/mL, controls = 221.22 ± 4.39 μg/mL, $p = 0.637$). *$p < 0.05$, n.s. = $p > 0.05$. Abbreviations: AD, Alzheimer's disease; CLU, clusterin; CSF, cerebrospinal fluid; n.s., not significant; SEM, standard error mean.

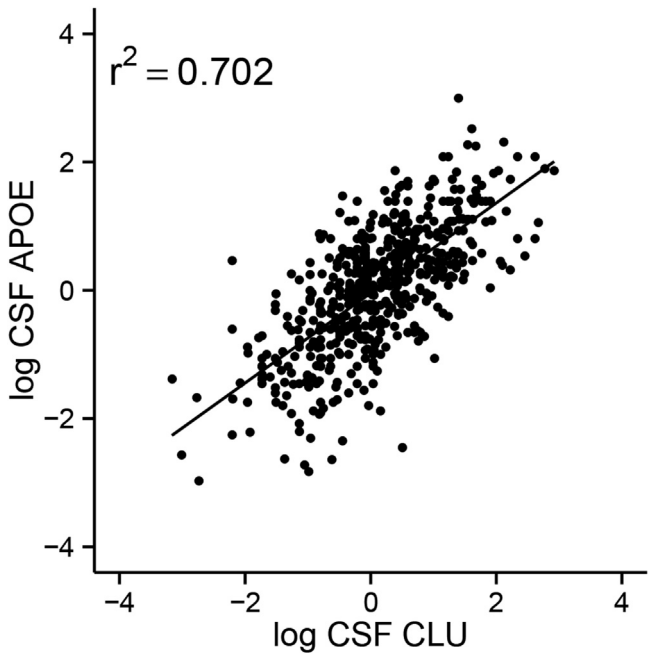

Fig. 2. Scatterplot of association of log normalized CSF levels of CLU and CSF levels APOE (r = 0.702, $p < 2.2 \times 10^{-16}$). Abbreviations: APOE, apolipoprotein E; CLU, clusterin; CSF, cerebrospinal fluid.

Table 3
Association of top SNPs with CSF CLU levels stratified by *APOE* carrier status

Chromosome	All samples					APOE ε4+ (n = 270)		APOE ε4− (n = 403)	
	SNP	Gene	*p*-value	Beta	MAF	*p*-value	Beta	*p*-value	Beta
16	rs2581305	*LINC00917*	3.98×10^{-7}	−0.608	0.052	5.83×10^{-4}	−0.615	4.03×10^{-4}	−0.577
2	rs12470837	3 kB from *LOC647996*	1.16×10^{-6}	−0.336	0.167	1.37×10^{-6}	−0.468	1.22×10^{-2}	−0.243
1	rs17507884	*MAGI3*	1.27×10^{-6}	−0.466	0.073	1.45×10^{-3}	−0.452	2.72×10^{-4}	−0.476
7	rs57375391	*LOC401312*	1.58×10^{-6}	−0.497	0.073	1.05×10^{-3}	−0.539	1.29×10^{-4}	−0.514
18	rs73431975	*SLC14A2*	1.70×10^{-6}	−0.573	0.056	1.91×10^{-2}	−0.489	8.20×10^{-5}	−0.584
8	rs10102274	*TMEM64*	2.27×10^{-6}	0.853	0.053	6.60×10^{-3}	0.673	7.54×10^{-5}	1.054
1	rs7533701	10 kB from *MAGI3*	2.46×10^{-6}	−0.358	0.133	1.09×10^{-2}	−0.307	3.37×10^{-4}	−0.357
3	rs417387	*VIPR1*	2.79×10^{-6}	−0.353	0.439	2.73×10^{-3}	−0.342	3.40×10^{-4}	−0.370
6	rs12195424	24 kB from *DST*	2.87×10^{-6}	−0.350	0.148	6.11×10^{-3}	−0.336	4.07×10^{-4}	−0.338
8	rs1693575	21 kB from *SNX31*	3.24×10^{-6}	0.363	0.318	4.63×10^{-4}	0.441	1.48×10^{-3}	0.328
4	rs1662046	*ADH1C*	3.30×10^{-6}	0.451	0.069	5.11×10^{-3}	0.447	8.68×10^{-5}	0.551
6	rs77121579	19 kB from *DST*	3.69×10^{-6}	−0.384	0.105	1.68×10^{-2}	−0.333	2.69×10^{-4}	−0.383
14	rs3783863	*FOXN3*	4.53×10^{-6}	−0.575	0.043	2.41×10^{-2}	−0.496	8.14×10^{-5}	−0.608
13	rs741668	12 kB from *ZC3H13*	4.64×10^{-6}	0.267	0.299	2.00×10^{-4}	0.302	7.49×10^{-3}	0.225
10	rs2456721	*SEC23IP*	4.94×10^{-6}	−0.266	0.286	2.60×10^{-3}	−0.265	6.67×10^{-4}	−0.266
13	rs1006064	*FAM124 A*	5.02×10^{-6}	−0.252	0.344	7.99×10^{-3}	−0.216	1.43×10^{-4}	−0.283
4	rs72635116	43 kB from *TLL1*	5.29×10^{-6}	−0.313	0.182	1.13×10^{-1}	−0.169	2.32×10^{-5}	−0.383
15	rs9972327	*IDH2*	5.62×10^{-6}	−0.400	0.223	1.74×10^{-5}	−0.531	1.47×10^{-2}	−0.299
2	rs79215379	*CTNNA2*	7.01×10^{-6}	−0.490	0.062	1.32×10^{-2}	−0.403	2.46×10^{-4}	−0.538
10	rs11006002	193 kB from *IPMK*	7.49×10^{-6}	−0.799	0.060	1.00×10^{-2}	−0.753	3.96×10^{-4}	−0.815
3	rs2442825	*SETD5*	7.79×10^{-6}	−0.237	0.487	1.03×10^{-2}	−0.191	4.75×10^{-4}	−0.261
21	rs9305339	110 kB from *LINC00314*	7.87×10^{-6}	0.264	0.394	9.22×10^{-4}	0.297	1.76×10^{-3}	0.245
8	rs17068510	*CSMD1*	7.96×10^{-6}	0.291	0.198	8.10×10^{-3}	0.250	3.41×10^{-4}	0.326
22	rs131814	*NCAPH2*	8.60×10^{-6}	−0.249	0.398	2.36×10^{-3}	−0.257	8.59×10^{-4}	−0.247
2	rs6758001	*LINC00607*	8.87×10^{-6}	0.638	0.034	1.71×10^{-2}	0.509	7.78×10^{-5}	0.769
7	rs144495862	*ABCA13*	9.22×10^{-6}	−0.422	0.198	1.17×10^{-3}	−0.545	2.80×10^{-3}	−0.363
7	rs1800795	*IL6*	9.94×10^{-6}	0.243	0.392	7.85×10^{-1}	0.024	7.40×10^{-8}	0.375

Key: APOE, apolipoprotein E; CLU, clusterin; CSF, cerebrospinal fluid; MAF, macrophage-activating factor; SNP, single nucleotide polymorphism.

significant SNP was rs2581305 on chromosome 16 in an intron of Long Intergenic Non-Protein Coding RNA 917 (*LINC00917)* (imputed, MAF 0.052, $p = 3.98 \times 10^{-7}$, beta = −0.608). The most significant genotyped SNP in LD was rs2581304 in an intron of *LINC00917* ($r^2 = 0.947$, D′ = 1, MAF 0.049, $p = 3.19 \times 10^{-6}$, beta = −0.575). Testing for SNP × APOE genotype interaction showed no significant effect ($p = 0.287$, beta = −0.346).

Because previous studies suggest an interaction with APOE and we found a strong correlation between CSF CLU levels and CSF APOE levels, we performed APOE genotype-stratified analyses. We did not find any genome-wide significant SNPs in the 270 ε4 carriers or 403 noncarriers (Table 3, Supplemental Figs. 3 and 4 respectively), but in ε4-individuals, 1 SNP almost reached genome-wide significance, rs1800795 on chromosome 7 in the promoter of *interleukin 6 (IL6)* (imputed, MAF 0.397, $p = 7.40 \times 10^{-8}$, beta = 0.375; Table 3, Supplemental Fig. 6). In the overall analysis (ε4+ and ε4− combined), this SNP was below the suggestive *p*-value ($p = 9.94 \times 10^{-6}$, beta = 0.243), whereas in the ε4+ group there was no suggestive association ($p = 0.785$, beta = 0.024). The most significant genotyped SNP in this locus in LD with rs1800795 was rs1800797 located within 2 kB of the 5′ end of *IL6* ($r^2 = 0.965$, D′ = 1, MAF 0.382, $p = 6.63 \times 10^{-7}$, beta = 0.241).

We did not find any genome-wide significant association in the GWAS for plasma CLU levels although the sample size was 20% greater than in CSF (n = 818, Table 4, Supplemental Fig. 5).We also found no correlation between the CSF and plasma GWAS results ($r^2 = -0.124$, Supplemental Fig. 6).

3.5. GO over-representation

In the ALIGATOR analysis of the CSF GWAS results, there was a significant excess of enriched categories: 190 categories with uncorrected $p < 0.05$ ($p = 0.051$), 77 $p < 0.01$ ($p = 0.016$), and 17 $p < 0.001$ ($p = 0.006$). Some categories with uncorrected $p < 0.001$ were related to wound healing and immune response (Supplemental Table 4) such as abnormal response to injury ($p = 5 \times 10^{-5}$), regulation of cytokine biosynthetic process ($p = 8 \times 10^{-5}$), cytokine pathway ($p = 1.80 \times 10^{-4}$), abnormal wound healing ($p = 2.2 \times 10^{-4}$), and delayed wound healing ($p = 3 \times 10^{-4}$). ALIGATOR analysis of the plasma GWAS showed there was no significant excess of enriched categories. There were 47 categories with uncorrected $p < 0.05$ ($p = 0.689$), 11 $p < 0.01$ ($p = 0.68$), and only 1 $p < 0.001$ ($p = 0.48$). Results for the plasma CPDB and PANTHER analyses can be found in Supplemental Table 5. Owing to the lack of association between plasma CLU levels and case-control status and the lack of correlation in the GWAS results between CSF and plasma, we focused our GO analyses on the CSF CLU results.

In the CPDB analysis of the pruned CSF GWAS results, there were 45 significant categories (Supplemental Table 6), and the PANTHER analysis resulted in 142 significant categories (Supplemental Table 7). There were 32 significant categories that overlapped in both analyses (Table 5). These categories were primarily related to lumenal side of membrane (CPDB $p = 1.02 \times 10^{-3}$, PANTHER $p = 2.08 \times 10^{-5}$), MHC protein complex (CPDB $p = 5.08 \times 10^{-4}$, PANTHER $p = 2.08 \times 10^{-5}$), wound healing (CPDB $p = 3.41 \times 10^{-2}$, PANTHER $p = 2.88 \times 10^{-4}$), coagulation (CPDB $p = 2.55 \times 10^{-2}$, PANTHER $p = 1.51 \times 10^{-4}$), hemostasis (CPDB $p = 2.82 \times 10^{-2}$, PANTHER $p = 1.64 \times 10^{-4}$), regulation of body fluid levels (CPDB $p = 2.55 \times 10^{-2}$, PANTHER $p = 3 \times 10^{-4}$), and positive regulation of immune system process (CPDB $p = 4.24 \times 10^{-2}$, PANTHER $p = 2.12 \times 10^{-4}$). Most of the significant categories shared between both analyses contained some of the major histocompatibility complex class I and class II genes (*HLA-A*, *HLA-G*, *HLA-DPB1*, *HLA-DPA1*), and several categories contained *IL6* (including wound healing, coagulation, hemostasis, regulation of body fluid levels, and positive regulation of immune system process). *IL6* was also involved in most of the categories with uncorrected $p < 0.001$ in the ALIGATOR analysis.

Table 4
Top SNPs ($p < 1 \times 10^{-5}$) from GWAS of association with plasma CLU levels (n = 818)

Chromosome	SNP	Gene	*p*-value	MAF
1	rs4428865	928 kB from *LOC102723336*	6.53×10^{-7}	0.224
3	rs12492269	*LINC01014*	2.22×10^{-6}	0.062
12	rs4930776	*ANO2*	3.20×10^{-6}	0.372
3	rs2007029	*GRM7*	3.94×10^{-6}	0.169
10	rs1575951	326 kB from *LINC01163*	4.01×10^{-6}	0.116
13	rs7995618	*LOC160824*	4.10×10^{-6}	0.118
12	rs66478310	5 kB from *LOC102723562*	4.86×10^{-6}	0.042
2	rs7589728	32 kB from *THNSL2*	4.95×10^{-6}	0.103
1	rs520885	24 kB from *MTF2*	5.04×10^{-6}	0.436
9	rs11793419	*PTPRD*	5.33×10^{-6}	0.306
6	rs2502399	36 kB from *LOC102724704*	5.35×10^{-6}	0.382
4	rs13121109	78 kB from *EDNRA*	6.40×10^{-6}	0.111
8	rs4545046	*LOC646843*	7.12×10^{-6}	0.379
3	rs2029773	16 kB from *LINC00635*	8.12×10^{-6}	0.473
8	rs4637816	86 kB from *LOC102724874*	9.31×10^{-6}	0.066
11	rs58655671	*OR51E2*	9.91×10^{-6}	0.172

Key: CLU, clusterin; GWAS, genome-wide association studies; MAF, macrophage-activating factor; SNP, single nucleotide polymorphism.

4. Discussion

In a previous study of 99 patients and 39 controls, CSF levels of CLU were significantly higher in AD patients ($p = 0.002$) (Nilselid et al., 2006). In our much larger study of 356 cases and 317 controls, we also found that CSF levels of CLU were significantly higher in cases than controls ($p = 0.03$), and we found CSF CLU levels were associated with tau/Aβ ratio ($p = 1.06 \times 10^{-7}$) which is highly predictive of cognitive decline. There was no difference in plasma CLU between cases and controls ($p = 0.114$) nor any association of plasma CLU levels with CSF tau/Aβ ratio ($p = 0.478$). In a different previous study, there was no association between plasma CLU levels and incidence of AD (n = 926, $p = 0.77$), which is consistent with our findings (Schrijvers et al., 2011). All these data suggest that CSF CLU levels would be more informative than plasma CLU levels for AD studies, although not as informative as CSF Aβ or tau.

We have previously shown that CSF APOE levels are an endophenotype for AD (Cruchaga et al., 2012), and animal studies suggest that APOE and CLU may interact and have an additive effect on Aβ levels to play a key role in AD. A previous study demonstrated that $CLU^{-/-}/APOE^{-/-}$ mice had increased amyloid deposition and significantly higher levels of soluble and insoluble Aβ40 and Aβ42 in the CSF ($p < 0.001$) than $CLU^{-/-}$ or $APOE^{-/-}$ alone (DeMattos et al., 2004). Additionally, Western blotting revealed that CLU levels were significantly lower in $APOE^{-/-}$ mice ($p = 1 \times 10^{-4}$) (DeMattos et al., 2004) suggesting that there could be an interaction between CLU and APOE. Here, we report for the first time that CLU and APOE levels are strongly correlated in the CSF of humans ($r^2 = 0.702$, $p < 2.2 \times 10^{-16}$) which indicates that CLU and APOE may interact in the human brain as well.

Previously, we successfully used CSF tau and phosphorylated tau levels in an endophenotype-based approach to find novel loci associated with AD (Cruchaga et al., 2013); thus, we decided to look for variants associated with CLU levels since CLU has also been strongly associated with AD. In our GWAS, we did not find any genome-wide significant SNPs associated with CSF or plasma CLU levels. Several SNPs had *p*-values $<10^{-5}$, so an increase in sample size may provide enough power to observe genome-wide significant SNPs in these loci. Interestingly, the GWAS of ε4-individuals (N = 403) had 1 SNP in *IL6* of borderline genome-wide significance ($p = 7.58 \times 10^{-8}$). This SNP is located in the promoter of *IL6*, and the −174C allele was previously associated with higher levels of

Table 5
Top gene ontology categories from CPDB ($p < 0.05$) and PANTHER ($p < 8.33 \times 10^{-3}$) analyses of CLU CSF GWAS results

GO ID	Function	Total genes	Candidate genes	CPDB *p*-value	PANTHER *p*-value
GO:0042611	MHC protein complex	28	4	5.08E−04	2.08E−05
GO:0098553	Lumenal side of endoplasmic reticulum membrane	28	4	5.08E−04	2.08E−05
GO:0071556	Integral component of lumenal side of endoplasmic reticulum membrane	28	4	9.96E−04	2.08E−05
GO:0098576	Lumenal side of membrane	28	4	1.02E−03	2.08E−05
GO:0030662	Coated vesicle membrane	123	6	1.88E−03	7.25E−05
GO:0042605	Peptide antigen binding	10	3	1.93E−03	2.70E−05
GO:0030658	Transport vesicle membrane	83	5	2.29E−03	1.12E−04
GO:0030666	Endocytic vesicle membrane	140	5	1.10E−02	1.19E−03
GO:0051240	Positive regulation of multicellular organismal process	517	12	2.02E−02	3.47E−05
GO:0042613	MHC class II protein complex	20	3	2.24E−02	2.07E−04
GO:0030176	Integral component of endoplasmic reticulum membrane	121	4	2.24E−02	4.88E−03
GO:0031227	Intrinsic component of endoplasmic reticulum membrane	126	4	2.35E−02	5.62E−03
GO:0050817	Coagulation	514	11	2.55E−02	1.51E−04
GO:0009611	Response to wounding	726	13	2.55E−02	2.17E−04
GO:0048518	Positive regulation of biological process	5128	46	2.55E−02	2.53E−04
GO:0050878	Regulation of body fluid levels	652	12	2.55E−02	3.00E−04
GO:0007010	Cytoskeleton organization	830	12	2.55E−02	2.38E−03
GO:0007596	Blood coagulation	514	11	2.82E−02	1.51E−04
GO:0007599	Hemostasis	519	11	2.82E−02	1.64E−04
GO:0030155	Regulation of cell adhesion	593	11	2.82E−02	5.06E−04
GO:0006996	Organelle organization	2854	30	3.19E−02	3.71E−04
GO:0016043	Cellular component organization	4847	41	3.19E−02	2.24E−03
GO:0042060	Wound healing	649	12	3.41E−02	2.88E−04
GO:0098552	Side of membrane	407	9	3.76E−02	4.86E−04
GO:0002694	Regulation of leukocyte activation	128	7	4.24E−02	8.64E−06
GO:0002684	Positive regulation of immune system process	285	8	4.24E−02	2.12E−04
GO:0001817	Regulation of cytokine production	254	7	4.24E−02	5.90E−04
GO:0048878	Chemical homeostasis	826	13	4.24E−02	7.30E−04
GO:0003281	Ventricular septum development	44	3	4.24E−02	2.00E−03
GO:0048522	Positive regulation of cellular process	4400	38	4.24E−02	2.41E−03
GO:0050865	Regulation of cell activation	138	7	4.56E−02	1.40E−05
GO:1902589	Single-organism organelle organization	1895	21	4.69E−02	1.77E−03

Key: CLU, clusterin; CPDB, ConsensusPathDB; CSF, cerebrospinal fluid; GO, gene ontology; GWAS, genome-wide association studies; PANTHER, Protein Analysis THrough Evolutionary Relationships.

IL6 in the brain (Licastro et al., 2003). IL6 has been associated with diffuse plaques in the brains of AD patients (Hull et al., 1996). IL6 was also reported to induce hyperphosphorylation of tau (Quintanilla et al., 2004). IL6 appears to play a role in modulating expression of CLU (Pucci et al., 2009), and *CLU* small interfering RNA knockdown increased IL6 baseline production in fibroblast-like synoviocytes ($p < 0.05$) (Devauchelle et al., 2006). Together, these data suggest that IL6 may play a role in AD pathology, possibly by regulating CLU levels or by CLU modulating IL6 levels.

Additionally, our GO analyses of the CSF GWAS results included categories related to wound healing and immune response indicating that CSF levels of CLU may be associated with wound healing and immune response, not only through IL6 but also other genes. IL6 is important in wound healing, not only through proinflammatory effects but also by promoting cell migration (Ebihara et al., 2011; Nasole et al., 2014). Previous research demonstrates that CLU may also play a role in healing, particularly in the brain. CLU improved healing after brain ischemia in wild-type mice where there was an increase in CLU messenger RNA in astrocytes in the peri-infarct area up to 3 months postischemia, and $CLU^{-/-}$ mice showed a significant reduction in healing postischemia (Imhof et al., 2006). Further research is necessary to explore the potential role of CLU in response to tissue damage and how this may influence the progression of AD.

It is interesting to note that the significant GO categories in our CSF and plasma analyses were very different. Whereas CSF levels of CLU may be associated with response to tissue damage, plasma levels of CLU may be associated with channel and transporter activity. Although there does not currently appear to be a strong association between CLU expression and channel or transporter activity, CLU was found to be important in recovery of hypofunctioning salivary glands, possibly by influencing the expression of *aquaporin-5* and 2 receptors involved in protein secretion (Mishima et al., 2012). *Aquaporin 1* was one of the genes mapped to SNPs with $p < 1 \times 10^{-4}$ in the plasma GWAS results and most of the other mapped genes were receptors, channels, or subunits.

In conclusion, we have demonstrated that CSF levels of CLU are significantly associated with powerful endophenotypes for AD (tau, ptau, and Aβ42) as well as AD status and CSF tau/Aβ ratio indicating that CSF CLU levels may be a good phenotype to use when studying AD. We also found a strong correlation between CSF levels of CLU and APOE which supports previous research that indicates there may be an important interaction between these 2 proteins or a common pathway that influences Aβ. Our genetic analyses suggest that the role of CLU in AD may not be limited to Aβ. Although there were no genome-wide significant hits in any of our GWAS, we did find several SNPs with suggestive *p*-values in our analyses of CSF CLU levels including 1 SNP in *IL6* that almost reached genome-wide significance. IL6 is related to immune response but also response to tissue damage. Our GO analyses indicate CLU may also be related to wound healing as well as immune response, further research is necessary to determine this.

Disclosure statement

Kelly Bales and Eve H Pickering work for Pfizer. The other authors have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.neurobiolaging.2015.09.009.

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